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X-RAY INACTIVATION OF PHOSPHOFRUCTOKINASE

THE RELATIVE RADIATION SENSITIVITY OF THE CATALYTIC AND THE ALLOSTERIC PROPERTIES

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SUMMARY

Rabbit muscle phosphofructokinase has been irradiated in solution with X-rays.

The dose–response curve for the loss of catalytic activity was biphasic, indicating that the enzyme consists of two fractions of widely differing sensitivity to radiation. Sucrose gradient studies indicated that the resistant fraction was associated with high molecular weight aggregates of the enzyme. The sensitive fraction was found to be inactivated with a G value of 0.055. Measurements of the disappearance of SH groups upon irradiation and of the protective effect of *p*-chloromercuribenzoate blocking prior to irradiation indicated that destruction of SH groups plays an important role in the radiation-induced loss of the catalytic activity.

The allosteric activity, as measured by the ability of AMP to reverse the ATP inhibition of the enzyme, was relatively resistant to radiation inactivation. Loss of allosteric activity did not occur until a considerable threshold dose had been accumulated. The shape of the dose–response curve could be explained by the radiation-induced changes in kinetic parameters. It was shown that the radiation-induced decrease in allosteric activity was due to a reduced sensitivity to ATP inhibition in the irradiated enzyme.

INTRODUCTION

Previous studies have shown that the radiation inactivation of sulfhydryl enzymes can be accounted for largely by destruction of their SH groups[1–4]. In the few cases where the effect of ionizing radiation on the allosteric properties of enzymes has been studied, the allosteric function has proved to be more sensitive to irradiation than the catalytic function[4–6]. From a radiobiological point of view it is of consider-

Abbreviations: DTNB, 5,5′-dithio-bis-(2-nitrobenzoic acid); PCMB, *p*-chloromercuribenzoate.

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able interest to establish whether this is the case for regulatory enzymes in general. In order to throw light on this question we have studied the X-ray inactivation of the sulfhydryl enzyme phosphofructokinase (ATP:D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11). A radiation study of this enzyme seemed of interest for two reasons. Phosphofructokinase is a key enzyme in the regulation of glycolysis. Furthermore, recent studies have shown that blocking of its sulfhydryl groups has no effect on the allosteric properties of the enzyme, even under conditions giving extensive inactivation of the catalytic activity[7].

MATERIALS AND METHODS

Materials

Rabbit muscle phosphofructokinase, aldolase, triose phosphate isomerase and α-glycerophosphate dehydrogenase were obtained from Boehringer and Soehne Mannheim, Germany. The phosphofructokinase had a specific activity of 40–50 μmoles/min per mg of protein[8]. The barium salt of fructose 6-phosphate (Schwartz Bio Research, Orangeburg, N.Y.) was converted to the sodium salt. AMP, ATP, NADH, *p*-chloromercuribenzoate (PCMB), 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) and Cleland's reagent were obtained from Sigma Chemical Co., St. Louis, Mo.

Enzyme assay

The assay was carried out at room temperature. The fructose 1,6-diphosphate formation was measured by converting the product enzymatically to α-glycerophosphate. The enzymatic reduction of this compound was measured by recording NADH oxidation at 340 mμ.

The standard assay mixture, slightly modified from LING, MARCUS AND LARDY[8], contained in a total volume of 3 ml: 33 mM Tris–HCl (pH 8); 2 mM fructose 6-phosphate; 2 mM ATP; 4 mM $MgSO_4$; 10 mM Cleland's reagent; 5 mM KCl; 0.16 mM NADH and an excess of aldolase, triose phosphate isomerase and α-glycerophosphate dehydrogenase. The reaction was initiated by the addition of phosphofructokinase. When indicated, the enzyme was preincubated with an excess of Cleland's reagent for 5 min at room temperature prior to the assay, and the reaction was started by adding the rest of the assay mixture. The ratio of $MgSO_4$ to ATP was kept constant at a value of 2. The absorbance was read at 1-min intervals in a Zeiss spectrophotometer. A linear decrease in absorbance was found after an initial lag period. The slope of the linear part of the curve was taken as a measure of the enzyme activity. The assays were performed at room temperature.

The effect of the allosteric modifier, AMP, was determined by assaying the enzyme activity at pH 6.7 (Tris–HCl) in the presence of inhibiting concentrations of ATP (3 mM) and low concentrations of fructose 6-phosphate (0.2 mM), as previously described[7]. The allosteric activity is expressed as the percent increase in the activity observed in the presence of 1 mM AMP.

The protein concentration was determined by the method of LOWRY *et al.*[9].

Density gradient centrifugation

The enzyme was sedimented in a 5–25% sucrose gradient in 20 mM Tris–HCl buffer, pH 8.0, containing 1 mM Cleland's reagent. It was centrifuged in a SW-39

rotor of Spinco model L for 18 h at 36 000 rev./min at 4°. 10-drop fractions were collected and assayed for enzyme activity and protein content.

Irradiation conditions

The enzyme suspension was centrifuged and the crystals dissolved in 20 mM phosphate buffer (pH 8.0). Unless otherwise stated, the enzyme concentration during irradiation was 100 μg/ml. The enzyme was irradiated in glass vials in the presence of air at 0° with a Stabilipan X-ray machine. The irradiation parameters were 220 kV, 20 mA and 0.5 mm Cu filter. The dosimetry was carried out with the Fricke dosimeter.

RESULTS

Inactivation of the catalytic activity

Irradiation of phosphofructokinase with increasing doses led to biphasic response curves (Fig. 1A). Initially the activity disappeared as an exponential function of the dose and then the curves levelled off. The shape of the dose–response curves suggested that the enzyme consists of two fractions differing in sensitivity by a factor

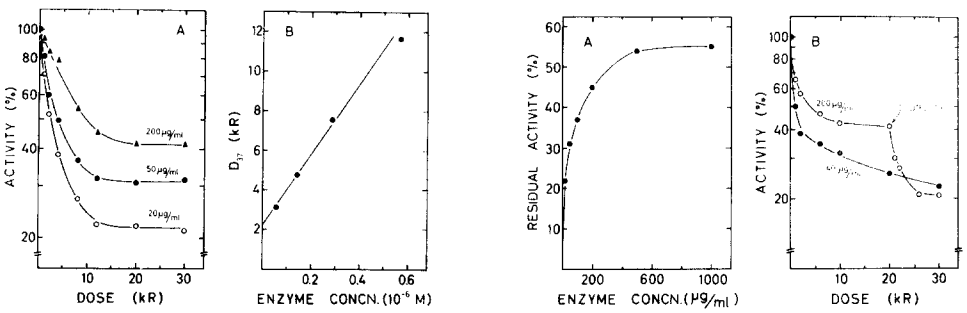


Fig. 1. X-ray inactivation of phosphofructokinase. A. Different concentrations of enzyme were irradiated in 20 mM phosphate buffer (pH 8.0) in equilibrium with air at 0°. The activity is expressed in percent of that of the unirradiated control. B. The D_{37} dose as a function of the enzyme concentration. The D_{37} doses were obtained from the initial, exponential part of dose inactivation curves. A molecular weight of 380 000 was used for phosphofructokinase[19].

Fig. 2. Effect of enzyme concentration on the radioresistant fraction. A. Residual activity determined after a dose of 30 kR, as a function of enzyme concentration. B. Effect of dilution on the dose–response curve.

of about 20. In Fig. 1B are plotted the D_{37} doses, obtained from the initial, exponential part of the dose–response curves, for increasing concentrations of the enzyme. A G value of 0.055 was obtained from the slope of the line.

The data in Fig. 1A indicated that the proportion of the more resistant fraction depends on the enzyme concentration. This view is supported by the data in Fig. 2A which demonstrate that with increasing enzyme concentration, the activity of the remaining resistant fraction increased up to about 50% of the initial activity. In order to study whether the resistant fraction was formed by a radiation-induced process, a relatively concentrated solution of enzyme was irradiated until the activity levelled off, and it was then diluted 5-fold with buffer. When this diluted sample was

further irradiated, it again showed a biphasic response curve (Fig. 2B). The final activity of the sample was the same as that of another sample which was irradiated at the same concentration as the diluted sample. The results indicate that the enzyme consists of a mixture of relatively radiation-sensitive molecules and a more resistant fraction existing in reversible equilibrium in solution. The data indicate that the resistant fraction was not formed during the irradiation.

Since preparations of phosphofructokinase are known to consist of a mixture of molecules of different size[10], it was conceivable that the radiation sensitivity of the enzyme may be related to its state of aggregation. In order to study this question, native and irradiated enzyme was subjected to centrifugation in a sucrose density gradient, and the activity of the various fractions was measured. It is seen from Fig. 3 that in the native enzyme the major part of the enzyme activity was present in a slow-moving, light fraction and a smaller part in a fast-moving, heavier fraction. Upon irradiation drastic changes occurred in the activity pattern. Thus, after a radiation dose which was sufficient to remove all the activity of the sensitive fraction

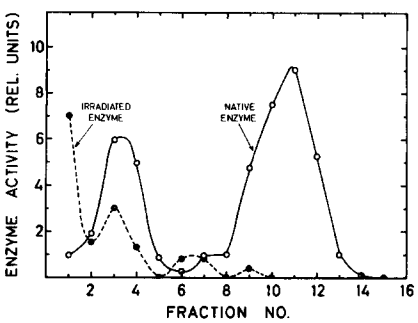


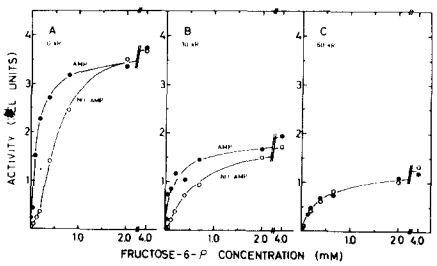
Fig. 3. Sedimentation profiles of native and irradiated phosphofructokinase in a sucrose density gradient. The enzyme (1 mg/ml) was irradiated with 120 kR (27% remaining activity). Gradien density centrifugation and enzyme assay were carried out as described in MATERIALS AND METHODS

and approx. 50% of that of the more resistant fraction, it was found that the enzyme activity associated with the light fraction had disappeared almost completely. In contrast, the activity associated with the heavy fraction was only moderately reduced. The results indicate that the resistant fraction consists of aggregate forms of the enzyme. It was found that the content of the resistant fraction varied somewhat in the different enzyme preparations and that it appeared to increase upon ageing. This supports the above interpretation, as it is known that aggregation of the enzyme increases under such circumstances[11]. The conclusion that the higher forms of aggregation are more resistant to irradiation appears reasonable, as it might be expected that radiosensitive groups would be partly covered on aggregation.

Inactivation of the allosteric activity

Phosphofructokinase is inhibited by high concentrations of one of its substrates, ATP[12–14]. This inhibition is reversed by AMP[15], and it appears that the ratio ATP/AMP is the important factor in the regulatory function of the enzyme[16,17]. When the enzyme activity is measured at pH below neutrality in the presence of 3 mM ATP as a function of increasing fructose 6-phosphate concentration, a sigmoidal

response curve is obtained, as shown in Fig. 4A. When AMP is added, the saturation curve changes into a hyperbolic response curve, which resembles that found at low ATP concentrations by other authors[17]. The effect of irradiation on the saturation curves for fructose 6-phosphate are shown in Figs. 4B and C. It is seen that the maximum velocity decreased upon irradiation and that the affinity of the enzyme for the substrate, both in the presence and absence of AMP, decreased. At high radiation doses the two curves seem to coincide, and the results suggest that the sigmoidal nature of the curves obtained in the absence of AMP vanishes upon irradiation. This latter point is confirmed in Fig. 5, where the data have been plotted according to Hill. The results demonstrate that with increasing radiation doses, the kinetics changed from second to first order. Concurrently, the apparent K_m increased by a factor of 2. In the presence of AMP, the reaction was first order with respect to the substrate, both in the native and the irradiated enzyme. However, here the effect of irradiation on K_m was greater.



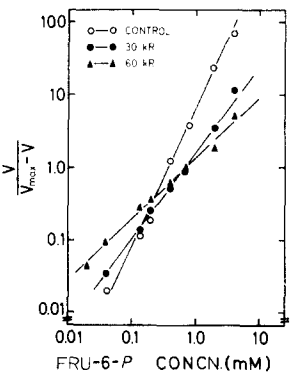


Fig. 4. Effect of irradiation on fructose 6-phosphate saturation curves. The enzyme was irradiated at a concentration of 100 μg/ml. Enzyme activity was measured in the presence of 3 mM ATP at pH 6,7 ,in the presence and absence of 1 mM AMP.

Fig. 5. HILL plots for rate *versus* fructose 6-phosphate concentration for native and irradiated phosphofructokinase. Data taken from Fig. 4. ○, native enzyme; $n = 1.8$; $[S_{0.5}] = 0.4$ mM. ●, enzyme irradiated with 30 kR; $n = 1.2$; $[S_{0.5}] = 0.6$ mM. ▲, enzyme irradiated with 60 kR; $n = 0.9$; $[S_{0.5}] = 0.7$ mM.

In the present paper the allosteric activity has been expressed as the percent activity increase in the presence of 1 mM AMP when the activity is measured at pH 6.7 at high ATP (3 mM) and low fructose 6-phosphate (0.2 mM) concentrations. In Fig. 6A the effect of radiation on the activity measured in the presence and absence of AMP is shown. It is seen that, at zero dose, AMP increased the enzyme activity about 4-fold. Upon irradiation, the activity initially decreased rapidly, both when measured in the presence and absence of AMP. However, the activity in the absence of AMP levelled off more rapidly, with the consequence that at high radiation doses, the two curves approached each other, implying that the allosteric function was then lost.

The relative radiation sensitivity of the catalytic and the allosteric function is apparent from Fig. 6B where, for both functions, the remaining activity, expressed

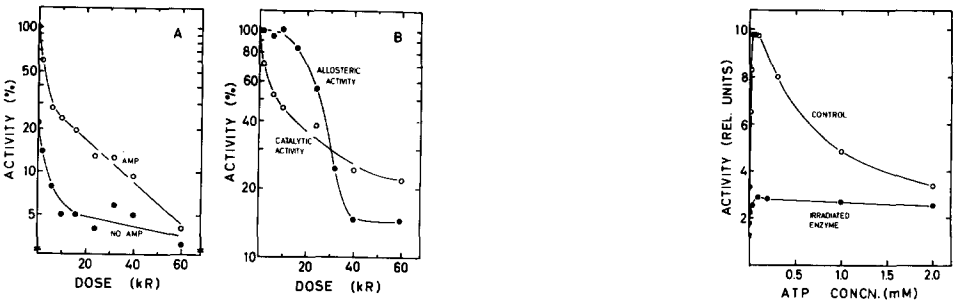


Fig. 6. Effect of irradiation on allosteric activity of phosphofructokinase. A. Loss of activity as a function of radiation dose, measured in the presence of 3 mM ATP at pH 6.7, in the presence and absence of 1 mM AMP. B. Loss of catalytic and allosteric activity as a function of dose. Catalytic activity measured at pH 8.0. The allosteric activity is expressed as the ability of AMP to stimulate the activity, calculated in percent of that of the unirradiated sample. The results are based on the data in A.

Fig. 7. Effect of irradiation on ATP inhibition of phosphofructokinase. The enzyme activity was measured at pH 6.7 in the presence of increasing concentrations of ATP. The enzyme concentration was 100 μg/ml and the radiation dose was 120 kR. The activity of the control and the irradiated enzyme is expressed in the same arbitrary units. The specific activity of the unirradiated enzyme measured under standard conditions (pH 8) was 43 μmoles/min per mg protein.

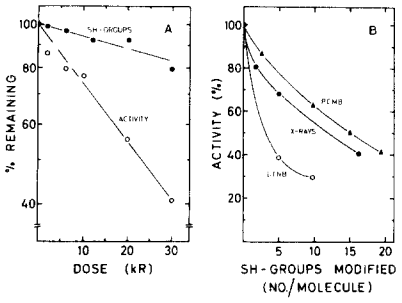
in percent of the initial activity, is plotted *versus* the radiation dose. It is seen that the allosteric activity was unaffected up to radiation doses where the catalytic activity was strongly reduced and largely the resistant fraction remained. Upon further irradiation, the allosteric function was rapidly lost.

The simplest explanation for the radiation-induced loss of the allosteric property, is that the radiation modifies the enzyme in such a way that its sensitivity to ATP inhibition is lost. That this is indeed the case is shown in Fig. 7. While in the native enzyme the activity decreased strongly when the ATP concentration exceeded 0.01 mM, a similar ATP inhibition was not found in the irradiated enzyme.

Role of sulfhydryl groups

It has previously been shown that blocking of the SH groups of phosphofructokinase leads to loss of the catalytic activity[7,18]. In order to elucidate the role of SH groups in the radiation inactivation of the enzyme, the concurrent SH destruction was measured and the extent of inactivation was compared with that obtained by sulfhydryl reagents. It is seen (Fig. 8A) that when the enzyme was irradiated until 50% of the activity remained, only 15% of the SH groups had disappeared. Since the enzyme contains about 70 SH groups per mol. wt. 380 000 (refs. 18, 19), this corresponds to the loss of 11–12 SH groups (Fig. 8B). The data in this figure also demonstrate that with DTNB, 50% inactivation was achieved when only about 3 SH groups were blocked, while with PCMB 15 SH groups had to be blocked. Clearly, per SH group altered, DTNB is far more effective in inactivating phosphofructokinase than are X-rays or PCMB. Presumably, this is related to the fact that DTNB is fairly specific in its action and reacts only with the more reactive sulfhydryl groups, while PCMB and X-rays are less discriminating in their reaction with sulfhydryl groups.

Although the data in Fig. 8B demonstrate that the X-ray destruction of



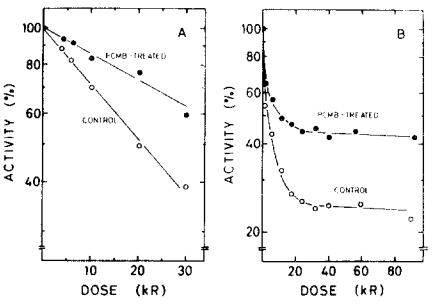


Fig. 8. Disappearance of enzyme SH groups upon irradiation. A. The enzyme in a concentration of 600 μg/ml was irradiated with increasing doses of X-rays. The enzyme activity was determined, and the remaining SH groups were titrated with PCMB in the presence of 5 M urea. B. Enzyme activity after alteration of SH groups. The data for DTNB and PCMB are taken from CHAPMAN, SANNER AND PIHL[7].

Fig. 9. Effect of PCMB treatment on the radiation sensitivity of phosphofructokinase. The enzyme was preincubated with 25 equivalents (A) and 15 equivalents (B) of PCMB prior to irradiation. After exposure all samples (PCMB-treated and controls) were incubated with Cleland's reagent for 5 min before the enzyme assay. A. Effect of PCMB treatment on the sensitivity of the radiosensitive fraction. Enzyme concentration 600 μg/ml. B. Effect of PCMB treatment on the radioresistant fraction of the enzyme. Enzyme concentration, 100 μg/ml.

enzyme SH groups is sufficiently extensive to account for the loss of enzyme activity, these results alone do not constitute proof that the radiation inactivation is caused by this effect. More conclusive evidence for the role of sulfhydryl groups in the X-ray inactivation was obtained in experiments in which the enzyme was irradiated after prior blocking of SH groups with PCMB. The results in Fig. 9A show that blocking of 25 SH groups with PCMB afforded definite protection (dose reduction factor of about 2) against the inactivation of the radiosensitive fraction of the enzyme. The effect of PCMB treatment on the radiation sensitivity of the resistant fraction is shown in Fig. 9B. It can be seen that in the PCMB-treated enzyme, the dose–effect curve tapered off at a higher level than in the untreated enzyme. These data suggest that PCMB treatment had two effects, *viz.* to protect the sensitive fraction and to displace the equilibrium in the direction of the resistant fraction. Previously PARMEGGIANI *et al.*[11] have reported that PCMB prevented aggregation of phosphofructokinase. However, in this case a 1000-fold higher concentration of PCMB was used than in the present studies.

DISCUSSION

The effect of ionizing radiation on phosphofructokinase differs in one important respect from that found in previous studies of allosteric enzymes. Thus, in phosphofructokinase the allosteric site is relatively resistant to inactivation by radiation, in contrast to the findings with aspartate transcarbamylase[5,6] and phosphorylase *b* (ref. 4). Recently we have found[20] that in fructose 1,6-diphosphatase as well, the allosteric site is less sensitive to X-rays than is the catalytic site. It thus appears that no generalization can be made concerning the relative radiation sensitivity of the catalytic and allosteric sites in regulatory enzymes.

In the SH enzymes previously studied, the radiation inactivation could largely

be accounted for by destruction of the sulfhydryl groups[1–4]. The present results demonstrate that in the case of phosphofructokinase as well, destruction of sulfhydryl groups plays an important role in the radiation-induced loss of catalytic activity. This follows from the fact that radiation inactivation was associated with extensive loss of SH groups and that blocking of a fraction of the SH groups with PCMB afforded appreciable protection. In fact, it can be calculated that the protection obtained was sufficiently large that the loss of activity on irradiation could well be due entirely to the destruction of SH groups. However, more information is needed concerning the role of the different SH groups of phosphofructokinase to establish whether this is indeed the case.

The loss of AMP activation of phosphofructokinase upon irradiation was found to be due to the fact that irradiation reduced the sensitivity of the enzyme to ATP inhibition. Previously we have found that the loss of allosteric activity on acetylation is likewise due to loss of ATP inhibition[7].

The loss of ATP inhibition of phosphofructokinase on irradiation can not be due to destruction of sulfhydryl groups since the allosteric function is not affected by sulfhydryl reagents. The loss of allosteric function of phosphofructokinase on acetylation occurred under conditions when primarily tyrosine groups are acetylated[7]. Since tyrosine belongs to that group of amino acids which are most extensively destroyed when proteins are irradiated in solution[21], it seems plausible that the radiation inactivation of the allosteric function of phosphofructokinase involves modification of tyrosine residues. The finding that acetylation of phosphofructokinase changes the order of the reaction with respect to the substrate in the same way as does irradiation supports the view that destruction of tyrosine residues may, at least in part, explain the loss of allosteric function.

The present finding that the allosteric function of phosphofructokinase was not affected until a considerable threshold dose had been given may at first sight seem surprising. It should be realized, however, that the allosteric property, as measured here, is a complex function, depending on several parameters. The studies of the effect of irradiation on kinetic parameters indicated that the order of the reaction, as well as the apparent K_m, initially varied linearly with the radiation dose. It can be shown that if, on the basis of the kinetic parameters measured, theoretical curves are constructed for the disappearance of the allosteric activity as a function of the radiation dose, these curves have, in principle, the same shape as that actually observed. The peculiar dependence of the loss of allosteric function on the dose of irradiation can therefore be satisfactorily explained by the changes induced in kinetic parameters, and it is not necessary to invoke other explanations.

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